ISSN: 2833-0064

Research Article

Journal of Biopolymers and Theoretical Studies

Fractionation of Fish Waste Oil through Low-Temperature Crystallization in Acetone Followed by Urea Complexation

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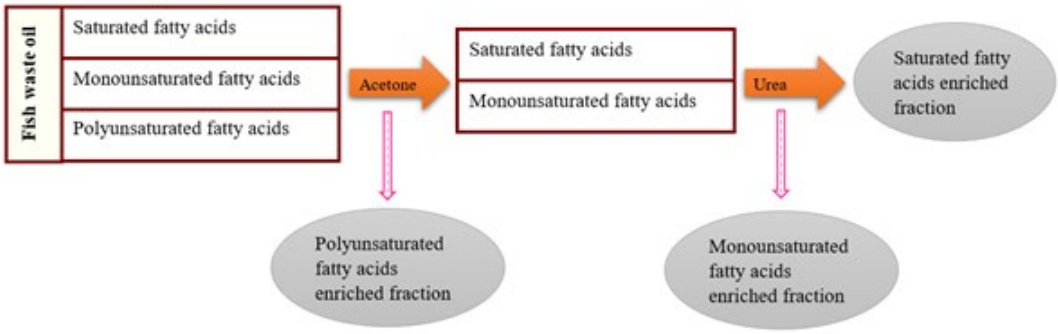
Submitted: 15 Nov 2022; **Accepted:** 30 Nov 2022; **Published:** 26 Dec 2022

Citation: Rastegari, H., Espootin, S., Ghaziaskar, H, S., Yatim, I, N., & Ali, N. (2022). Fractionation of Fish Waste Oil through Low-Temperature Crystallization in Acetone Followed by Urea Complexation. *J Biop Theo Stud,* 2(1), 71-78.

Abstract

Fatty acids fractionation has enabled the introduction of many fatty acids into new food/feed applications. Yet, a simple, efficient, and low-cost process to separate biomass oily feedstocks into valuable fatty acids fractions remains a challenge. Herein, a new, efficient, and simple fractionation method is developed to obtain enriched polyunsaturated fatty acids fraction from fish waste oil using acetone for crystallization at low-temperature followed by urea complexation. This method obtained two fractions, one enriched in polyunsaturated fatty acids, and the other enriched in saturated fatty acids. The fraction enriched in polyunsaturated fatty acids contained oleic, eicosapentaenoic, and docosahexaenoic acids with mass percentages of 58, 20, and 22%, respectively. While the fraction enriched in saturated fatty acids contained 78, 12, 5, and 5 wt. % palmitic, myristic, oleic, and stearic acids. The solvent was recovered from the liquid phase with an efficiency of 80% and reutilization of it obtained almost identical results.

Graphical Abstract



Keywords: Fractionation, Fish waste oil, Oleic acid, Palmitic acid, Solvent crystallization, Urea complexation.

Introduction

Simultaneous world population growth and food-related industries have led to a remarkable increase in the production of bio-waste. Seafood waste, especially fish waste, is one of the most important types of bio-waste that discarding to the environment creates huge economic and environmental worries. With the growing attention to the circular economy, proper valorization of these wastes is a central issue to be resolved [1]. Fish waste is a cheap and available source of health-promoting polyunsaturated fatty acids (PUFAs) [2]. Fish oil contains polyunsaturated fatty acids (PUFAs) such

as oleic acid (C18:1), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6) which are widely used as nutraceuticals ingredients. There are several health benefits behind the consumption of PUFAs. The presence of PUFAs in the diet is mandatory for proper nerve function, blood clotting, brain health, and muscle strength [3]. One of the most recent significant potentials uses is in COVID-19 therapy because of its ability to lower the risk of thrombosis [4]. However, there are several issues associated with the direct addition of PUFAs to foodstuff including its typical fishy smell, unpleasant taste, contamination with heavy metals, poor oxidation stability and most importantly the high content of saturated fatty acids (SFAs) such as myristic acid (C14:0) and palmitic acid (C16:0) [5,6]. Generally, the content of these PUFAs in different types of fish oils is less than 60 wt.%. Hence, for the application of this type of oil in the formulation of healthy food, it is mandatory to reduce the content of SFAs [7,8]. The other advantage of separating SFAs is that these fatty acids could be used as surfactants with different applications such as the treatment of wastewater [9].

To date various methods have been developed and introduced for PUFA enrichment. Winterization, molecular distillation, low-temperature crystallization in different solvents, urea complexation, open-column chromatography, enzymatic hydrolysis, and supercritical carbon dioxide are the most common available methods which have been used for PUFAs fractionation either alone or in combination with other methods [10-14]. However, for commercial aspects, the enrichment should be performed via efficient, low costs, sustainable, and clean processes with maximum extraction yields and optimal lipid profile [15]. The traditional method, known as urea complexation, fractionates saturated and monounsaturated fatty acids from PUFAs [16]. Simplicity, robustness, reproducibility, low cost and investment, non-expensive reagents, and the possibility of urea recycling are some of the most important advantages accompanied with this method [17]. Taking these advantages into account, scaling for industrial applications is easy [18]. This technique is based on the formation of complexes between urea and linear hydrocarbon chains. Capability of urea molecules in the ethanolic solution to form hexagonal cavities is the principal factor behind this enrichment. These hexagonal cavities form channels that trap SFAs through Van der Waals forces [19]. The geometry of the hydrocarbon chain affects the stability of the complex where the most stable complexes are formed by SFAs followed by monounsaturated fatty acids. Whereas the PUFAs linear chain does not generate strong enough interactions with urea. This difference in tendency to form complexes with urea led to the formation of a solid-liquid system. SFAs and monounsaturated fatty acids complex with urea remain in the solid fraction while PUFAs remain in the liquid fraction [16,17]. To this end, the solid phase contains monounsaturated fatty acids other than SFAs.

Urea complexation technique resulted in the separation of mixtures of fatty acids according to their degree of unsaturation. Saturated and monounsaturated fatty acids have an almost linear structure and hence can be easily trapped by the cavity of the urea complex that is known as the most important disadvantage of this process. This is due to the fact that the separated fraction must reach a certain level of purity to further explore their potential application in nutrition and pharmaceutics [15,20,21]. Therefore, combination of two or more fractionation method is mandatory. Low temperature crystallization resulted in the separation of PUFAs from more SFAs because of their various degrees of unsaturation and molecular configurations [22]. Most studies in the field of low temperature crystallization have focused on the utilization of hexane and acetone as solvents [23-26]. However, utilization of acetone result in more selective fractionations due to its smaller molecular volume [24]. As far as we know, low-temperature crystallization in acetone followed by urea complexation has not been reported for fractionating fish waste oil.

The present study has focused on using a preparation method before urea complexation which is based on different solubilities of monounsaturated fatty acids, PUFAs, and SFAs in acetone as solvent. First, crystallization of fatty acids in low-temperature in acetone was carried out. Then urea complexation was used in order to obtain two fractions, one enriched in PUFAs, and the other one enriched in SFAs. The objective is to develop a simple low-cost and efficient method to obtain PUFAs concentrates from fish waste oil and other type of oily biomass.

Materials and Methods

Materials

The fish waste oil was supplied by Arman Jonoub Co. (Iran). Normal hexane, methanol (purity >99%), and absolute ethanol were obtained from Iran Chemicals Co. (Iran), Shiraz Petrochemical Co. (Iran), and Bidestan Co. (Iran), respectively. Table 1 represents the fatty acid composition of the fish waste oil. NaOH (purity ≥98%), urea (purity >99%), HCl (purity 37%), acetone (purity >99%) were purchased from Sigma-Aldrich (Germany), Merck (Malaysia), Sigma-Aldrich (Germany), and Sasol Co. (South Africa), respectively.

Table 1: Fatty Acid Composition of Fish Waste Oil used in this Study.

Fatty acid (wt.%)	Formula	Result
Myristic acid	C14:0	10.3±3.4
Palmitic acid	C16:0	38.5±5.8
Oleic acid	C18:1	28.6±6.3
Stearic acid	C18:0	7.7±3.9
Eicosapentaenoic acid	C20:5	6.2±0.2
Docosahexaenoic acid	C22:6	8.8±3.1

Methods

Hydrolysis of Fish Waste Oil to FFAs by Saponification

The starting oil contains triglycerides that are not able to interact with urea to form complexes. Hence, saponification was used to hydrolyze triglycerides into their corresponding FFAs that are able to form complexes with urea [27]. First, 25g of oil was mixed with 20 mL of ethanol (96 wt.%) and then 5.65 g of KOH was added to the mixture. Then the mixture was refluxed at 85°C under nitrogen atmosphere. After a duration of 1h, the reaction was stopped through addition of 30 mL distilled water and 30 ml of n-hexane and then acidified with HCl solution (6M), adjusting pH to 2 and a separatory funnel was used in order to obtain two distinct phases. Washing with water was used to remove polar impurities from FFAs phase and this washing step was repeated for three times. Each time 30 mL of distilled water was applied for liquid-liquid extraction. After that the two phases was separated completely at room temperature through centrifugation at 6000 rpm for 15 min. Finally, a rotary evaporator was used for drying FFAs under vacuum at 40°C. The final FFA content of this phase was higher than 95%.

Urea Complexation Process

FFAs mixture obtained in the Hydrolysis of Fish Waste Oil to FFAs by Saponification was used as feedstock at this step and the fractionation process was performed under the conditions reported by Vazquez, et al. with some modifications [28]. First 3g of FFAs mixture was weighted and mixed with different amounts of urea in a 120 mL flask containing 20 mL of ethanol. Then the mixture temperature was increased to 65°C and stirred at 350 rpm for complete solvation of urea and cooled immediately by immersing the flask in water bath. After that the mixtures remained for 24 h at different temperatures including -5, 0, 5, 25°C. Then, the mixture was filtered using a Büchner Flask under vacuum equipped with a filter funnel with porosity of 70–100 µm. For efficient separation of fatty acids four successive washing steps with normal hexane was used and 2 mL of solvent was used at each step. Consequently, two phases including one solid and one liquid phase obtained. The solid phase composed of urea and the fatty acids that formed complexes while the liquid phase contained the solvents and the fatty acid that did not form complexes with urea. Recovery of the fatty acids that formed complex with urea was performed through washing with hot distilled water at temperature of 60°C and mass ratio of 1:1 wt.%. For complete solvation of the solids the sample pH was adjusted at 2 via the addition of hydrochloric acid (6M). Then, two extractions were carried out with normal hexane as solvent and 10 mL of solvent was used at each extraction. Finally, hexane was removed via vacuum distillation in a rotary evaporator at 60°C. The final product was enriched in SFAs and MUFAs. Purification of the liquid phase was performed via washing the liquid twice with distilled water in a weight ratio of 40%. These washing steps are mandatory to purify the final product through removing any polar impurity especially urea. Finally, hexane was vacuum distilled at aforementioned conditions to obtain the final product concentrated in PUFAs.

Low-Temperature Crystallization in Acetone followed by Urea Complexation Process

5g of FFAs mixture was dissolved in 50 ml of acetone and the solution was stored at a temperature of -10°C for 24 hours. The two phases obtained were separated through centrifugation at 6000 rpm for 15 min at -10°C. The liquid phase was vacuum dried in a rotary evaporator, at 35°C to obtain a rich fraction of PUFAs. While the solid phase contained SFAs and monounsaturated FAs went under urea complexation for the more enrichment in palmitic acid as mentioned in Urea Complexation Process It should be mentioned that throughout this study each experiment was performed in triplicate.

Analysis of FFAs by GC

Enriched fractions were analyzed directly by GC-FID without using any sample preparation step. A GC-FID system (model 3420, BEIFEN, China) was used for analysis. The capillary column was an HP-5 (30m, 0.32 mm i.d., and 0.25 µm film thickness). An injection volume of 1 µL and a 20:1 split ratio was used. The injector and the detector temperature were set at 315°C and 325°C, respectively. Temperature programming was used for analysis with an initial temperature of 70°C. The oven temperature remained for 2 min at this temperature, then increased to 310°C at a rate of 20 °C.min-1, and hold at 310°C for 5 min. Exit of all species from the column is the principal factor behind remaining column temperature for 5 min at 310°C. Samples were quantified through internal standard and peak area normalization method. The peak area of a non-precipitating compound in the reactions was selected as internal standard. The peak area of each precipitating compound was used to calculate the concentration of that compound [29]. The concentration of each fatty acid was calculated using the following

equation:

Concentration (wt.%)=(Fatty acid weight (mg))/(Fraction weight (mg))×100

Results and Discussions

Table 1 presents the fatty acid composition of the feedstock while the chemical structure of them is presented in Figure 1. Considering the weight percent of fatty acids and also their applications, this study is focused mainly on the enrichment of palmitic acid from the fish waste oil.

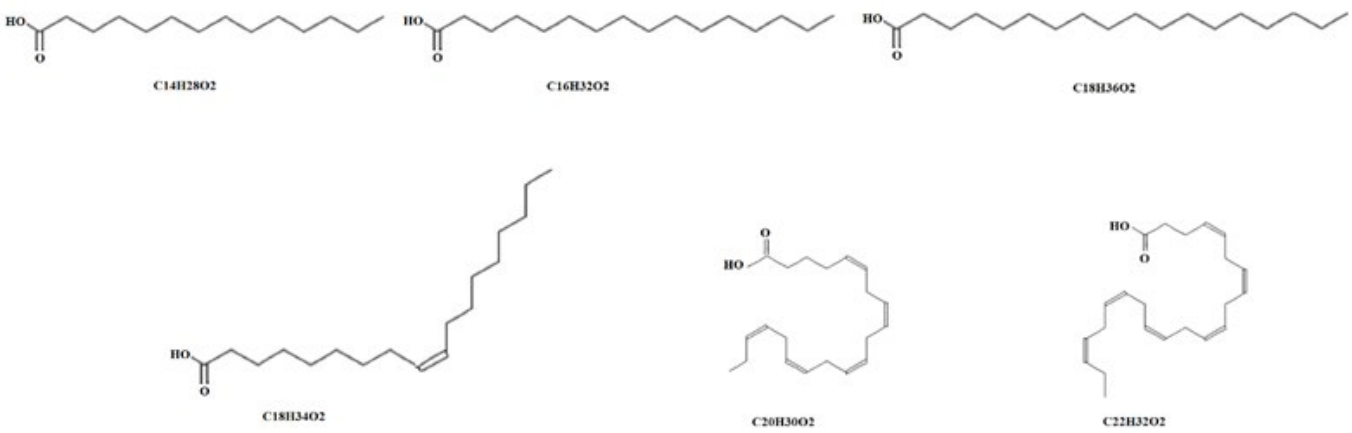


Figure 1: Chemical Structure of Fatty Acids presents in the Fish Waste Oil Sample.

The main fatty acids present in the selected fish waste oil feedstock are palmitic acid (C16:0) and oleic acid (C18:1) with mass percent's of 39% and 29%, respectively. Palmitic acid is saturated while oleic acid is a monounsaturated fatty acid which both could react with urea and consequently separated from feedstock as a concentrated fraction. Hence, the urea complexation method was first optimized and the effect of crystallization temperature and mass ratio of urea to fatty acids for a duration of 24h were investigated. Then low-temperature crystallization in acetone followed by urea complexation was investigated at optimum conditions. Acetone was selected as the solvent due to the different solubility of palmitic acid and oleic acid in it [30].

Figure 2 shows the effect of temperature on the fractionation efficiency using urea complexation method. Saturated fatty acid molecules (myristic and palmitic) have capability to form complexes with urea more readily than the PUFAs [31]. Strictly speaking, the high restriction in the urea crystal occurs for PUFA molecules. As a result, they cannot move freely in the tunnel which result in smaller uptake capacity [32]. Almost similar results were observed at temperatures of 0°C and 5°C. The best results were obtained at -5°C. At these conditions myristic, palmitic, oleic, and stearic acids formed urea complexes with values of 11, 57, 26, and 5%, respectively. While the mass percent of both eicosapentaenoic and docosahexaenoic acids were lower than 1%.

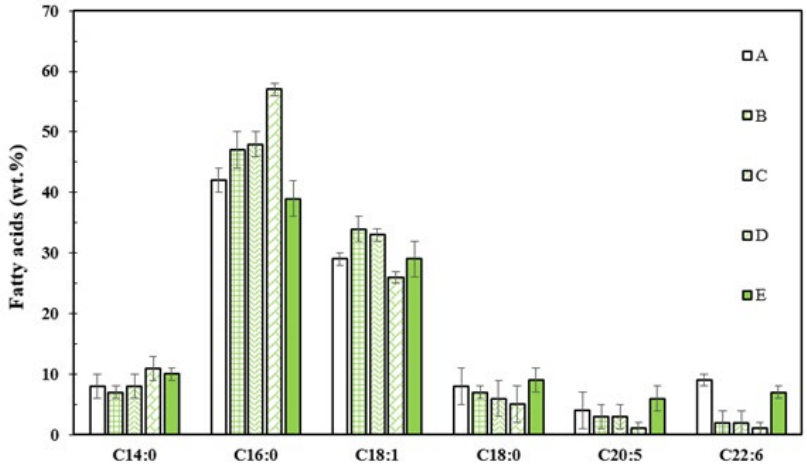


Figure 2: Effect of temperature on the fractionation efficiency using the urea complexation method; 25°C (A), 5°C (B), 0°C (C), -5 °C(D), and fish waste oil (E). Urea complexation time was 24h and urea to the fatty acid weight ratio of 3:1.

The most abundant SFAs have straight hydrocarbon chains which makes them possible to form complexes with urea. While the bend structure of PUFAs molecules due to the presence of two or more double bonds in their hydrocarbon chains, make them impossible to form adducts with urea molecules Figure 1. However, in the case of mono-unsaturated fatty acids (MUFAs), the only carbon-carbon double bond in the hydrocarbon chains mostly has a cis-configuration which makes the molecule diameter bigger than SFAs. These MUFAs could compete with SFAs to block the urea channels [32].

The other important variable is temperature which affect the solubility of urea in ethanol. Selected temperature for this study was -5, 0, 5, 25°C. At room temperature the prepared urea solutions in ethanol are saturated. Temperatures below room temperature encourage the solutions to the supersaturated conditions. Using low temperatures especially direct cooling in the refrigeration, resulted in fast and easy complex formation of SFAs with urea and obtaining less concentration in non-urea complex fraction [32].

Figure 3 shows the effect of urea to fatty acid weight ratio on the fractionation efficiency using urea complexation method. The increase of urea complexation yield with weight ratio from 2.5 to 3.0 is evident. However, more increase in the weight ratio to 3.5 doesn't have significant effect on the process yield. Generally, the formation of the urea complex depends on the degree of unsaturation of fatty acids. Saturated long-chain molecules (C18:0) could easily form complex with urea as it is large enough to coordinate the aliphatic chain by urea [31]. This provides higher tendency to complex and crystalize eventually compared to the relatively smaller molecules such as C16:0 leaving behind the unsaturated fatty acids dominated in the solution. The presence of double bonds in the fatty acids chain will increase the molecule size and hence reduces the tendency of the complexation [31].

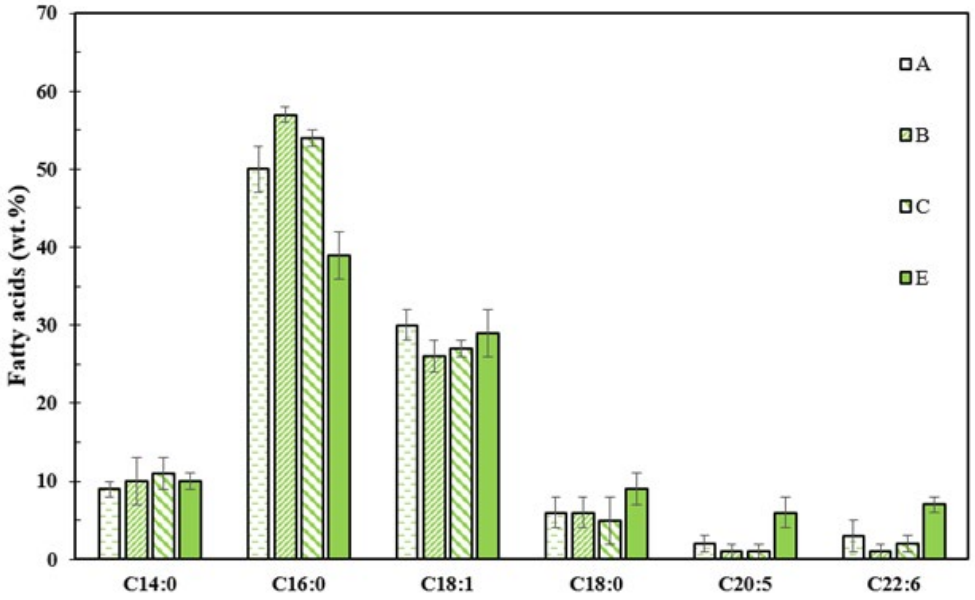


Figure 3: Effect of urea to fatty acid weight ratio on the fractionation efficiency using the urea complexation method; 2.5 (A), 3.0 (B), 3.5 (C), and fish waste oil (E). Urea complexation time was 24h and complexation temperature of -5°C.

Urea to fatty acid ratio is another effective variable in the urea complexation process. At low concentration of urea, the number of fatty acids molecules are higher than urea ones. Since, some fatty acid molecules could not be adducted. Consequently, a low yield for urea complexation obtains. By increasing urea concentration, the number of urea molecules in the system increase, resulting in the formation of adducts with fatty acids, leading to an increase in the yield of urea complexation. For a urea to fatty acid ratio of 2.5:1, the C20:5 and C22:6 concentration in the urea complex was lower than 5%, while for higher ratios of 3.0:1 and 3.5:1 these values reported to be 2%, and 3%, respectively, while the amount of saturated and mono-unsaturated fatty acids in the urea adducts increase. As mentioned before, both SFAs and MUFAs could easily form complexes with urea. However, greater bending degree of PUFAs resulted in their larger diameters and hence their adduction with urea is more difficult. Relative high melting points as well as low solubility in acetone are the main results for presence of C20:5 and C22:6 with amounts lower than 5% in the urea adducts after crystallization at a low temperature [20]. The maximum enrichment of fatty acids in this study was achieved with urea to fatty acid ratios of 3:1 and more. Since, the SFAs and MUFAs contents had not a significant difference in the range of 3:1-3.5:1, the ratio of 3:1 was selected as the optimum one. Selection of ratio of 3:1 as the optimum value for further studies help to decrease the amount of urea consumption.

In order to increase the efficiency of the separation of palmitic acid from oleic acid a novel method developed using acetone as solvent for crystallization at low-temperature followed by urea complexation [22]. Acetone was selected as crystallization solvent before urea complexation due to different solubility of palmitic acid and oleic acid is this solvent. Oleic acid is soluble in acetone while palmitic acid has a negligible solubility in acetone [33]. Figure 4 shows the effect of low-temperature crystallization in acetone followed by urea complexation on the fractionation efficiency.

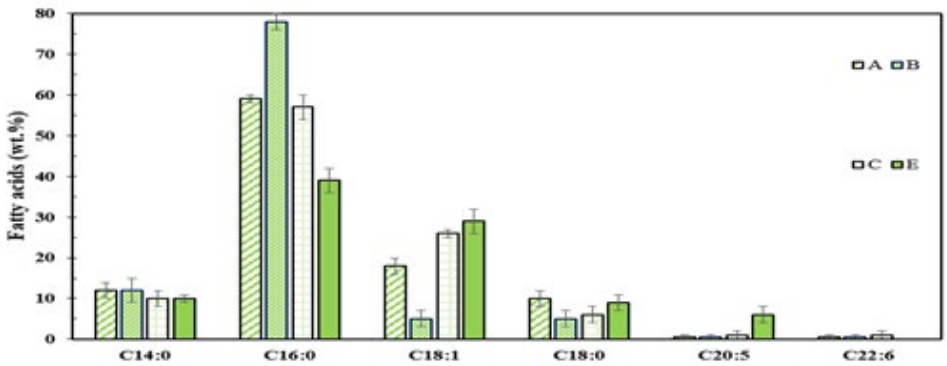


Figure 4: Effect of low-temperature crystallization in acetone followed by urea complexation on the fractionation efficiency; acetone (A), acetone and urea (B), urea (C), and fish waste oil (E).

As shown in Figure 4, the percentage of palmitic acid has increased to 78% using low-temperature crystallization in acetone followed by urea complexation. While using only urea complexation obtained a value of 57%. These results showed that the low-temperature crystallization in acetone followed by urea complexation is an effective method to increase the separation efficiency of oleic acid from palmitic acid. The results confirm that the experimental conditions are appropriate for the fractionation of palmitic acid.

The solvent recovery and composition were determined after one cycle. The solvent was recovered from the liquid phase through evaporation with an efficiency of 80%. This mass loss could be related to the solvent loss in the condensation step. The composition of recovered solvent was analyzed by GC-FID and only one peak was detected which was related to the solvent. Reutilization of the solvent obtained almost identical results.

Conclusions and Future Remarks

An efficient and simple procedure was developed for enrichment of palmitic acid from fish waste oil. This procedure is a consecutive process in which acetone was used as solvent for crystallization at low-temperature followed by urea complexation. Acetone was used as solvent in the low-temperature crystallization step due to different solubility of oleic acid and palmitic acid in it. SFAs fraction is rich in palmitic acid (78%) and contain 12wt. % meristic acid. While the other one contains oleic acid, eicosapentaenoic acid, and docosahexaenoic acid. The solvent was recovered with an efficiency of 80% and reutilized once with almost identical results. The rich fraction in palmitic acid can be used as surfactants. While the rich fraction in oleic acid and stearic acid can be used in food industries. In this way it is possible to increase fish processing industries' incomes through extending profit margins as a result of fish waste oil valorization as well as reducing the environmental impacts.

Authors Contribution

Hajar Rastegari: Conceptualization, Investigation, Formal analysis, Validation, Methodology, Writing original draft, review and editing.
Simin Espootin: Conceptualization, Investigation, Formal analysis, Validation, Methodology.
Hassan S. Ghaziaskar and Noraaini Ali: Review and Editing.
Norhafiza Ilyana Yatim: Preparing Figures.

Acknowledgments

Authors are thankful to Isfahan University of Technology for partial financial support.

References

1. Coppola, D., Lauritano, C., Palma Esposito, F., Riccio, G., Rizzo, C., & de Pascale, D. (2021). Fish waste: From problem to valuable resource. Marine drugs, 19(2), 116.
2. Al-Hilphy, A. R., Al-Mtury, A. A. A., Al-Shatty, S. M., Hussain, Q. N., & Gavahian, M. (2022). Ohmic Heating as a By-Product Valorization Platform to Extract Oil from Carp (Cyprinus carpio) Viscera. Food and Bioprocess Technology, 15(11), 2515-2530.
3. Innes, J. K., & Calder, P. C. (2020). Marine omega-3 (N-3) fatty acids for cardiovascular health: an update for 2020. International journal of molecular sciences, 21(4), 1362.
4. Djuricic, I., & Calder, P. C. (2021). Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: an update for 2021. Nutrients, 13(7), 2421.
5. Stevens, J. R., Newton, R. W., Tlusty, M., & Little, D. C. (2018). The rise of aquaculture by-products: Increasing food production, value, and sustainability through strategic utilisation. Marine Policy, 90, 115-124.
6. Vasile, F. E., Romero, A. M., Judis, M. A., & Mazzobre, M. F. (2019). Physicochemical, nutritional, and stability aspects of

a meat product (gluteus medius) enriched with encapsulated fish oil in polyelectrolyte beads containing Prosopis alba exudate gum. Food and Bioprocess Technology, 12(4), 654-664.
7. Kuo, C. H., Huang, C. Y., Chen, J. W., Wang, H. M. D., & Shieh, C. J. (2020). Concentration of docosahexaenoic and eicosapentaenoic acid from cobia liver oil by acetone fractionation of fatty acid salts. Applied Biochemistry and Biotechnology, 192(2), 517-529.
8. Liu, Y., Zhang, W., Wang, K., Bao, Y., Regenstein, J. M., & Zhou, P. (2019). Fabrication of gel-like emulsions with whey protein isolate using microfluidization: Rheological properties and 3D printing performance. Food and Bioprocess Technology, 12(12), 1967-1979.
9. Rashid, T. U., Kabir, S. F., Biswas, M. C., & Bhuiyan, M. R. (2020). Sustainable wastewater treatment via dye–surfactant interaction: a critical review. Industrial & Engineering Chemistry Research, 59(21), 9719-9745.
10. Ferrentino, G., Ndayishimiye, J., Haman, N., & Scampicchio, M. (2019). Functional activity of oils from brewer's spent grain extracted by supercritical carbon dioxide. Food and bioprocess technology, 12(5), 789-798.
11. González-Fernández, M. J., Ramos-Bueno, R. P., Rodríguez-García, I., & Guil-Guerrero, J. L. (2017). Purification process for MUFA-and PUFA-based monoacylglycerols from edible oils. Biochimie, 139, 107-114.
12. Kulkarni, N. G., Kar, J. R., & Singhal, R. S. (2017). Extraction of flaxseed oil: a comparative study of three-phase partitioning and supercritical carbon dioxide using response surface methodology. Food and Bioprocess Technology, 10(5), 940-948.
13. Liu, S., Zhang, C., Hong, P., & Ji, H. (2006). Concentration of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of tuna oil by urea complexation: optimization of process parameters. Journal of food engineering, 73(3), 203-209.
14. Magallanes, L. M., Tarditto, L. V., Grosso, N. R., Pramparo, M. C., & Gayol, M. F. (2019). Highly concentrated omega-3 fatty acid ethyl esters by urea complexation and molecular distillation. Journal of the Science of Food and Agriculture, 99(2), 877-884.
15. Rodríguez-González, I., Díaz-Reinoso, B., & Domínguez, H. (2022). Intensification Strategies for the Extraction of Polyunsaturated Fatty Acids and Other Lipophilic Fractions From Seaweeds. Food and Bioprocess Technology, 1-20.
16. Vázquez, L., Sánchez-Moyano, M., de la Iglesia, L., Reglero, G., & Torres, C. F. (2022). A new urea adducts method for PUFA concentration using green food grade solvents and avoiding ethyl carbamate formation. Food Chemistry, 392, 133197.
17. González-Fernández, M. J., Fabrikov, D., Lyashenko, S., Ferrón-Carrillo, F., & Guil-Guerrero, J. L. (2020). Highly concentrated very long-chain PUFA obtainment by Urea complexation methodology. Environmental Technology & Innovation, 18, 100736.
18. Shahidi, F., & Wanasundara, U. N. (1998). Omega-3 fatty acid concentrates: nutritional aspects and production technologies. Trends in food science & technology, 9(6), 230-240.
19. Smith, A. E. (1952). The crystal structure of the urea–hydrocarbon complexes. Acta Crystallographica, 5(2), 224-235.
20. Wang, X., Xiaohan, W., Chen, Y., Jin, W., Jin, Q., & Wang, X. (2020). Enrichment of branched chain fatty acids from lanolin via urea complexation for infant formula use. Lwt, 117, 108627.
21. Wang, X., Zhang, Z. J., Li, H. Z., Hou, T. Y., Zhao, Y. N., & Li, H. (2022). Urea complexation combined with rapid preparative reversed-phase liquid chromatography to separate α-linolenic acid from perilla seed oil: Purity, yield, and oxidation stability. Industrial Crops and Products, 187, 115473.
22. Mu, H., Zhang, H., Li, Y., Zhang, Y., Wang, X., Jin, Q., & Wang, X. (2016). Enrichment of DPAn-6 and DHA from Schizochytrium sp. oil by low-temperature solvent crystallization. Industrial & Engineering Chemistry Research, 55(3), 737-746.
23. Guil-Guerrero, J. L., López-Martínez, J. C., Rincón-Cervera, M. A., & Campra-Madrid, P. (2007). One-step extraction and concentration of polyunsaturated fatty acids from fish liver. Journal of the American Oil Chemists' Society, 84(4), 357-361.
24. Hamm, W. (1986). Fractionation-With or without Solvent?. Fette, Seifen, Anstrichmittel, 88(S1), 533-537.
25. Vázquez, L., & Akoh, C. C. (2012). Enrichment of stearidonic acid in modified soybean oil by low temperature crystallisation. Food Chemistry, 130(1), 147-155.
26. Fabritius, D. (2009). Mixtures of triglycerides of natural polyunsaturated fatty acids with high polyunsaturated fatty acid content, method for producing same and use thereof: Google Patents.
27. Vázquez, L., & Akoh, C. C. (2011). Concentration of stearidonic acid in free fatty acid and fatty acid ethyl ester forms from modified soybean oil by winterization. Journal of the American Oil Chemists' Society, 88(11), 1775-1785.
28. Vázquez, L., Ortego, E., Corzo-Martínez, M., Reglero, G., & Torres, C. F. (2018). Stearidonic acid concentration by urea complexation from Echium oil. Journal of Oleo Science, 67(9), 1091-1099.
29. Espootin, S., Sameti, M., & Zaker, S. (2021). Biodiesel from fish waste oil: synthesis via supercritical methanol and thermodynamic optimization. Clean Energy, 5(2), 187-195.
30. Mgbechidinma, C. L., Zheng, G., Baguya, E. B., Zhou, H., Okon, S. U., & Zhang, C. (2023). Fatty acid composition and nutritional analysis of waste crude fish oil obtained by optimized milder extraction methods. Environmental Engineering Research, 28(2).
31. Dovale-Rosabal, G., Rodríguez, A., Contreras, E., Ortiz-Viedma, J., Muñoz, M., Trigo, M., ... & Espinosa, A. (2019). Concentration of EPA and DHA from refined salmon oil by optimizing the urea–fatty acid adduction reaction conditions using response surface methodology. Molecules, 24(9), 1642.
32. Setyawardhani, D. A., Sulistyo, H., Sediawan, W. B., &

Fahrurrozi, M. (2015). Separating poly-unsaturated fatty acids from vegetable oil using urea complexation: The crystallisation temperature effects. J. Eng. Sci. Technol, 10, 41-49.
33. Calvo, B., Collado, I., & Cepeda, E. A. (2009). Solubilities of palmitic acid in pure solvents and its mixtures. Journal of Chemical & Engineering Data, 54(1), 64-68.